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Sialic Acid in Human Serum and Cerebrospinal Fluid

Comparison of methods and reference values

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Summary: Sialic acid was estimated simultaneously by three methods:
(A) chemical determination based on *Warren*'s method (Meth. Enzymol. *6*, 463–464 (1963)) with slight modifications,
(B) enzymatic measurement with a commercially available test kit, and
(C) high performance liquid chromatography (HPLC) according to *Silver* et al. (J. Chromatogr. *224*, 381–388 (1981)).

These methods showed closely correlated ($r > 0.930$) results and displayed similar precision data. Interference studies demonstrated sufficient specificity for the chemical assay, which was 5–6 times more sensitive than the enzymatic test and hence chosen for the establishment of reference values.

From 249 sera from healthy people between 16 and 63 years the 0.025–0.975-reference intervals were calculated to be 1.57–2.63 mmol/l for 127 men, and 1.69–2.64 mmol/l for 122 women with no significant dependence on age and sex. From 43 cerebrospinal fluids from healthy adults the respective values were 17.3–50.4 µmol/l. These data correspond to those of the literature.

Some chemical assays employing thiobarbituric acid were compared. They proved reliable in contrast to the reaction of serum with 4-dimethylaminobenzaldehyde.

Sialinsäure im menschlichen Serum und Liquor cerebrospinalis
Methodenvergleich und Referenzwerte

Zusammenfassung: Sialinsäure wurde mit drei Methoden vergleichend bestimmt:
(A) Chemische Bestimmung nach *Warren* (Meth. Enzymol. *6*, 463–464 (1963)) mit geringen Modifikationen,
(B) enzymatische Messung mit einer kommerziellen Testpackung und
(C) Hochleistungsflüssigkeitschromatographie (HPLC) nach *Silver* et al. (J. Chromatogr. *224*, 381–388 (1981)).
Die Ergebnisse dieser Verfahren waren eng korreliert ($r > 0{,}930$) und zeigten ähnliche Werte bei Untersuchung der Präzision. Störversuche ergaben eine ausreichende Spezifität für die chemische Bestimmung, die fünf- bis sechsfach empfindlicher als der enzymatische Test war und daher zur Ermittlung der 2,5–97,5%-Bereiche eingesetzt wurde.

Bei der Untersuchung von 249 Seren gesunder Erwachsener zwischen 16 und 63 Jahren betrugen sie, ohne signifikante Abhängigkeit vom Alter und Geschlecht, 1,57–2,63 mmol/l für 127 Männer und 1,69–2,64 mmol/l für 122 Frauen. Im Liquor cerebrospinalis von 43 gesunden Erwachsenen lagen die entsprechenden Konzentrationen zwischen 17,3 und 50,4 µmol/l. Alle Werte entsprechen den Angaben in der Literatur.

Wir untersuchten die Eigenschaften einiger Thiobarbitursäure-Verfahren, die im Gegensatz zur Reaktion von Serum mit 4-Dimethylaminobenzaldehyd als zuverlässig anzusehen sind.

Introduction

In addition to its elevation in cases of acute inflammation, rheumatoid arthritis and myeloma (1), sialic acid in serum has been widely discussed as a marker of tumour burden for more than 20 years (2–10). However, several methods for its determination have been recommended with different reference values, which serve as a basis for clinical interpretations. Therefore, this communication compares the ability of various methods to determine sialic acid (N-acetylneuraminic acid) reliably in human sera and describes the establishment of reference intervals.

Materials and Methods

Apparatus, samples and reagents

We recorded the absorbance spectra with a Uvikon 860 spectrophotometer (Kontron AG, Zürich, Switzerland) and measured all assays with a Hg-line photometer 6114 S Eppendorf (Netheler & Hinz, Hamburg, Germany). High performance liquid chromatography (HPLC) was performed with Kontron instruments. All procedures were carried out with micro equipment of Scientific Manufacturing Industries (Berkeley, CA 94710; micropettors) and Eppendorf (micro test tubes 3812, multipette 4780).

Sera from venous blood (n = 78) were taken at random for comparison of HPLC, enzymatic and chemical methods. Another 249 sera from apparently healthy adults (blood donors and volunteers) were analysed for the establishment of reference intervals. For this study we excluded sera with following criteria:

- glucose above 6.4 mmol/l (fasting) or above 8.0 mmol/l (postprandial) estimated by the glucose dehydrogenase method (11),
- creatinine above 110 µmol/l (males) and above 90 µmol/l (females) measured with alkaline picrate (12) by a kinetic method (13), and
- γ-glutamyltransferase above 28 U/l (males) and above 18 U/l (females) assayed with *L*-γ-glutamyl-3-carboxy-4-nitroanilide at 25 °C (14).

Cerebrospinal fluids (n = 43) were obtained by lumbar puncture for myelographia from patients in good health with normal values as follows:

- cell count less than 3/µl,
- protein below 500 mg/l determined with Coomassie Brilliantblue G-250 (15), and
- lactate below 2 mmol/l (enzymatic test, (16)).

Sialic acid determinations were done immediately after blood clotting or after storage intervals of 2–90 days at −28 °C, which did not alter the content of the specimens. We used N-acetylneuraminic acid from Fluka AG (Buchs, Switzerland) and Serva (Heidelberg, Germany) for the preparation of standards, while the test kit for the enzymatic method was purchased from Boehringer (Mannheim, Germany). The Aminex HPX-87 column was from Bio-Rad Laboratories (Richmond, CA 94804). Other chemicals were supplied by E. Merck (Darmstadt, Germany) of the highest purity available, and solutions were prepared with doubly distilled, deionized water. In the definitive chemical procedure we determined sialic acid by *Warren*'s method (17) using a micromethod (18) with slight modifications.

Procedures

The enzymatic determination (19) followed the instructions of the manufacturer. In this method N-acetylneuraminic acid is set free from glycoproteins by neuraminidase and converted to pyruvate by N-acetylneuraminic acid aldolase. The two subsequent steps comprise the generation of hydrogen peroxide and its final reaction with a substituted toluidine and 4-aminoantipyrine to form a 4-benzoquinone-monoimine dye via oxidative coupling (*Emerson* reaction). The determination with 4-dimethylaminobenzaldehyde (20) was performed according to *Shamberger* (10), and the otherwise discarded precipitates were washed with sodium chloride, 155 mmol/l, and ethanol, dried, weighed, then dissolved in 0.25 ml of sodium hydroxide, 2 mol/l. After dilution with 1.0 ml of water their absorbance was recorded at 546 nm. Then 0.2 ml of this solution was mixed with biuret reagent, centrifuged and measured for protein concentration (21).

In the initial step of all techniques based on the thiobarbituric acid reaction the samples were hydrolysed in sulphuric acid, 50 mmol/l (final concentration), for 60 min at 80 °C. For HPLC the hydrolysates were subsequently ultrafiltered through YMT membranes in micropartition systems MPS-1 (Amicon Corp., Danvers, MA 01923) by centrifugation at 1000 *g* for 15 min. The filtrate was chromatographed as described by *Silver* et al. (22). The protocol of the proposed procedure followed the modification of *Kattermann & Krieger* (18), but with a doubled volume of sodium arsenite to intensify the colour formation (tab. 1). To compare the sensitivity of different techniques, the respective absorbances were calculated to an assumed common volume of 1 ml (tab. 2). Assay of cerebrospinal fluid: 150 µl of the sample (150 µl of sodium chloride for the blank, 150 µl of N-acetylneuraminic acid, 16.2 µmol/l, as standard) were mixed with 50 µl of sulphuric acid, 200 mmol/l, before heating.

The check to specificity of the proposed chemical method, an equal volume of carbohydrate solution replaced the standard (proper reaction) or it was added to the standard assay (interference). For the comparison of serum sialic acid concentrations by different methods the results were subjected to standardized main component analysis (23).

Results

Chemical methods

A recommended simple method (10) for measuring sialic acid by reaction with 4-dimethylaminobenzaldehyde in hydrochloric acid did not achieve an end point. After 4 h, colour development for sera was linear with no perceptible plateau by 20 h, while standards displayed a different linear development of the blue chromophore during the first 9 h of reaction, with decreasing rates afterwards (fig. 1). The resulting triphenylmethane dye was pH-dependent and showed different spectra for sialic acid and serum. The shape of these spectra remained constant after the second hour of reaction for standards with a maximum at 528 nm (fig. 2). During the reaction of sera an additional second peak appeared at 575 nm, which became predominant after 20 h (fig. 3).

At pH 13 the spectrum equalled that of the dissolved precipitate, indicating some protein binding of the coloured product. There was no increase (within limits of ± 5%) of dry weight, protein and dye concen-

Tab. 1. Protocol for determining the concentration of N-acetylneuraminic acid in serum.

	Standard	Sample	Blank	Concentration in the actual reaction mixture,
	µl	µl	µl	mmol/l
Sialic acid, 1.617 mmol/l	5	—	—	
Serum	—	5	—	
Sodium chloride, 155 mmol/l	50	50	50	
Sulphuric acid, 67 mmol/l	150	150	150	50
Mix and incubate for 60 min at 80 °C. Cool to room temperature in tap water.				
Sodium *meta*-periodate, 200 mmol/l				48.8
in *ortho*-phosphoric acid, 9 mol/l	50	50	50	2195
Mix and incubate for 20 min at room temperature.				
Sodium *meta*-arsenite, 1.54 mol/l				760
and sodium sulphate, 500 mmol/l				247
in sulphuric acid, 50 mmol/l	200	200	200	43.7
Mix until the yellow colour of iodine has completely disappeared.				
Thiobarbituric acid, 37 mmol/l				20.4
in sodium sulphate, 500 mmol/l	500	500	500	380
Mix thoroughly, heat for exactly 15 min at 95 °C and cool to room temperature in tap water.				
Cyclohexanone	1000	1000	1000	
Agitate vigorously and centrifuge for 2 min at 10000 *g*				
Transfer the supernatant layer carefully into a cuvette and read the absorbance at 546 nm.				
Calculation: $c_{sample} = \Delta A_{sample} \times c_{standard}/\Delta A_{standard}$				

Tab. 2. Reaction conditions and sensitivity of various N-acetylneuraminic acid determinations with thiobarbituric acid according to the reference cited. Molar lineic absorbance of the chromophore (mean and standard deviation from 6 determinations) in organic solvents at 20 °C. For further details, see methods.

	Proposed method	*Kattermann & Krieger* (18)	*Warren* (17)	*Horgan* (26)	*Denny* et al. (25)
	Oxidation with periodate in mineral acids between pH 1.0 and 1.5				
Oxidizing equivalents, µmol	80	80	160	20	50
	Reduction with sodium arsenite			or thiosulphate	
Reducing equivalents, µmol	616	308	1540	24.6	50
Redox balance, µmol	−536	−228	−1380	−4.6	0
pH-value	1.90	1.20	2.42	2.34	1.54
Thiobarbituric acid, µmol	18.5	18.5	111	80	125
pH-value	1.98	1.58	2.45	2.50	2.78
Relative chromogenity	1.00	0.87	0.91	0.78	0.38
	Extraction with cyclohexanone			or acidic butanol	
pH-value (aqueous layer)	2.10	1.70	2.65	0.70	0.50
$\varepsilon_{546\,nm}$, m^2/mol (mean ± SD)	5976 ±208	5319 ±282	6422 ±316	4721 ±256	3048 ±655
Relative sensitivity	1.00	0.89	1.07	0.79	0.51

tration of the precipitate after the second hour of reaction. These findings pointed to a complete protein denaturation at this time. Tryptophan, deemed a chromogenic component (24), did not react. Obviously, all results were highly time-dependent with varying standard to sample ratios during the reaction course. Moreover, some protein binding of the product to be measured could be demonstrated.

All thiobarbituric acid-based assays showed a similar sequence of oxidation, reduction (mostly with an excess of arsenite), reaction and extraction below pH 2.5 followed by the formation of a red chromophore (λ_{max} 550 nm), which proved stable for at least 120 min. There was no correlation between colour intensity and redox balance. Doubling the amount of thiosulphate (in the technique of *Denny* et al. (25)) suppressed the reaction, whereas doubling the volume of the arsenite solution increased the absorbance by 15% compared with the original procedure (18) as shown in table 2. Arsenite cannot be replaced by selenite. To reduce the use of toxic arsenite and to improve the final extraction of the red chromophore, we tried in vain to replace 100 µl of this solution by potassium chloride, 1 mol/l. Although the relative absorbance of the coloured product already differs in the centrifuged aqueous mixture, the terminal extraction step essentially influences the qualities of the test. In this regard especially four points must be taken into consideration:

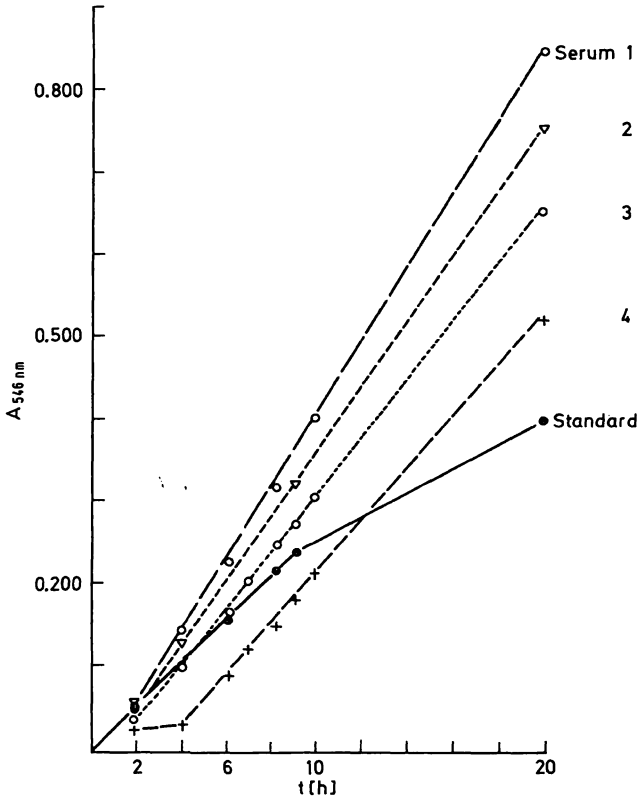

Fig. 1. Reaction of sera (broken lines) and sialic acid, 1.62 mmol/l (solid line), with 4-dimethylaminobenzaldehyde (according to *Shamberger* (10)) after various intervals measured at 546 nm.

- The molar lineic absorbance of the chromophore varies with kind, volume and temperature of the extracting solvent, but not with time, if the access of atmospheric moisture to hygroscopic solvents is prevented. Otherwise, slight, nearly invisible turbidities falsely enhance the signals recorded.
- At 20 °C acidic butanol only extracts 91–92% of the colour obtained with cyclohexanone. If the extractions are repeated, 6–9% yield of the first step is obtained with cyclohexanone and 13–18% with acidic butanol.
- The solvent volume should not be less than that of the aqueous phase: Reduction from 4.3 to 2.0 ml cyclohexanone in *Warren*'s (17) method diminishes the extracted colour from 100 to 68%.

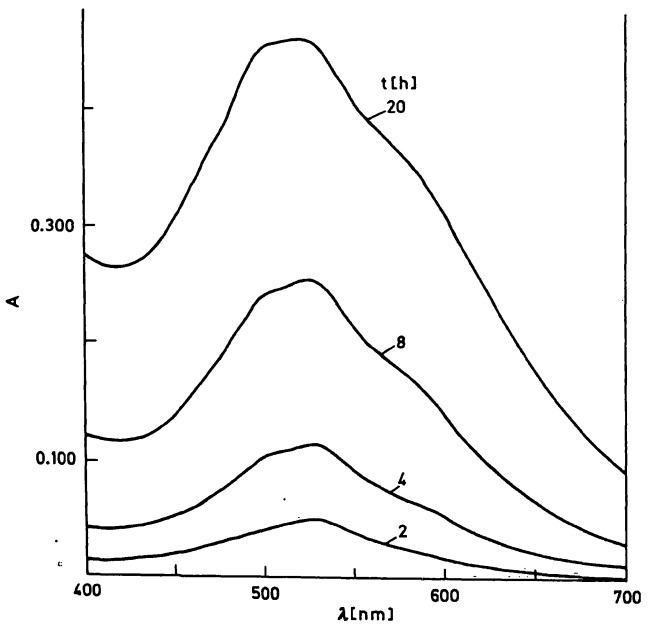

Fig. 2. Absorbance spectra of sialic acid, 1.62 mmol/l, reacting with 4-dimethylaminobenzaldehyde in hydrochloric acid (10) for 2, 4, 8, and 20 hours (versus blank).

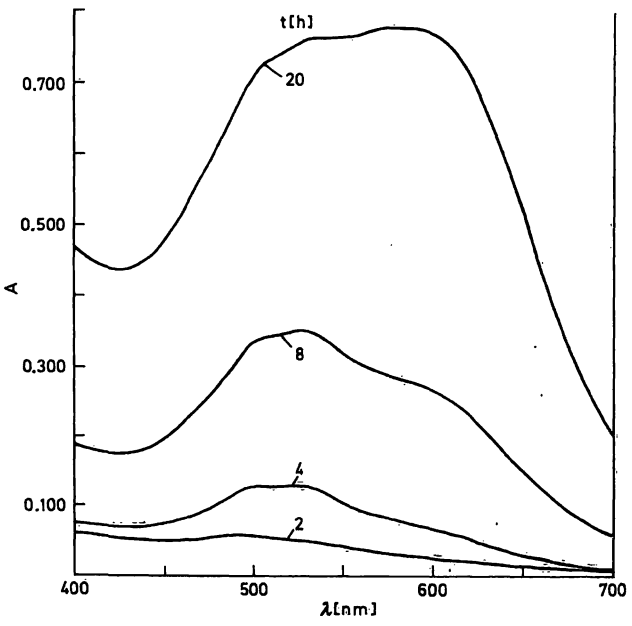

Fig. 3. Absorbance spectra of serum (supernatant according to *Shamberger* (10)) after reaction with 4-dimethylaminobenzaldehyde in hydrochloric acid for 2, 4, 8, and 20 hours (versus blank).

— The extraction with cyclohexanone does not significantly depend on temperature, but the molar lineic absorbance of the reaction product increases by about 5% from 37 to 20 °C. However, in acidic butanol the colour does not change with temperature, but the extraction at 20 °C yields 26% less chromophore than at 37 °C, which quantifies statements of *Horgan* (26).

Summarizing the aforementioned factors, we chose cyclohexanone and 20 °C for extraction from a turbid aqueous phase with high arsenite concentration (tab. 1), which evidently improved the sensitivity of the method (tab. 2), capable of quantitating less than 0.2 nmol of sialic acid. The analytical range was identical to the linear response of the original procedure (18). Standard curves for N-acetylneuraminic acid were stoichiometric from 30 µmol/l (9.3 mg/l) to 5 mmol/l (1547 mg/l) corresponding to absorbances of 0.014 to 1.430 at 546 nm. Thus, the chemical microassay was 5 to 6 times more sensitive than the commercial enzymatic test and well suited for the measurement of sialic acid in cerebrospinal fluid.

Accuracy and precision of the thiobarbituric acid method

The hydrolysis step reduced the colour formation from N-acetylneuraminic acid by 11.7 ± 0.08% (n = 5) which agreed fairly well with 9.5% reported from HPLC studies (22). Other pigments derived from various sugars were similarly diminished by the preceding influence of heat and acid. From sialic acid measurements in affinity chromatography we experienced the appearance of stable yellow pigments with 0.25 mol/l solutions of methyl-α-*D*-mannoside and N-acetyl-*D*-glucosamine. They maximally absorbed at 450 and 515 nm depressing the reaction of thiobarbituric acid with N-acetylneuraminic acid at 546 nm. These yellow chromophores, apparently being more polar, were less extracted by cyclohexanone than the product from sialic acid. Human serum albumin (40 g/l) caused no interference. In the presence of ethylene glycol, 0.5 mol/l, the reduced colour formation was accompanied by a white precipitate (soluble only in dimethylsulphoxide and sodium hydroxide, 2 mol/l) showing an infrared spectrum similar to that of glyoxal after reaction with thiobarbituric acid.

In pursuit of *Kuwahara*'s (27) studies on carbohydrate interference, but using cyclohexanone instead of acidic butanol extraction, we ascertained the effects of various sugars in concentrations, which were not sufficient to consume the periodate needed for complete oxidation of sialic acid. Table 3 contains the data of some reactions indicating a distinct interference by *L*-fucose (fig. 4) and confirming the relative stronger reaction of disaccharides (27). Their spectra exhibited maximal absorbance at 450 nm with a second but

Tab. 3. Reaction and interference of carbohydrates at 546 nm in the proposed modification of *Warren*'s method (17), related to the absorbance of sialic acid, 1.62 mmol/l. The figures for interference denote the deviation from this absorbance (taken as 1.000) caused by the compound concerned. For further details, see methods.

Substance and concentration	(mmol/l)	Reaction at 546 nm	λ_{max} (nm)	Interference at 546 nm	λ_{max} (nm)
Sodium chloride	155	0.000	—	1.000	550
N-Acetylneuraminic acid	1.62	1.000	550	—	—
α-*D*-Glucose	20	0.006	450	0.977	550
α-*D*-Glucosamine	20	0.005	450	1.020	550
N-Acetyl-*D*-glucosamine	20	0.005	450	1.000	550
Galactose	20	0.003	450	0.990	550
N-Acetyl-*D*-galactosamine	20	0.008	450	1.007	550
L-Fucose	20	0.000	450	0.640	550
	5			0.883	550
Fructose	20	0.003	450	0.981	550
D-Mannose	20	0.003	450	0.983	550
D-Xylose	20	0.002	450	0.977	550
D-Ribose	20	0.010	450	0.979	550
Sucrose	20	0.009	450	0.983	550
	5			1.000	550
Maltose	20	0.021	450 + 515	0.981	550
	5			0.990	550
Lactose	20	0.020	450 + 5145	0.973	550
	5			1.000	550
Methyl-α-*D*-mannoside	20	0.019	450 + 515	0.992	550

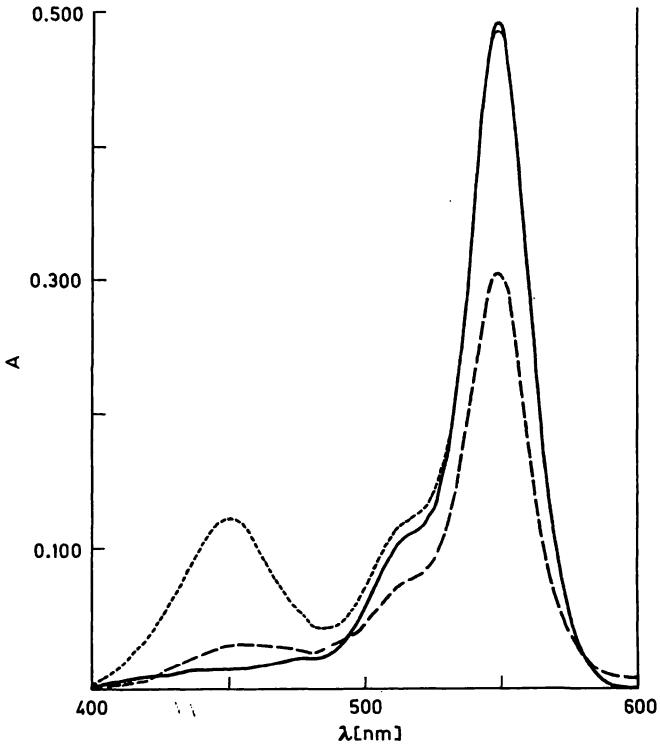

Fig. 4. Absorbance spectra of thiobarbituric acid-derived pigments from N-acetylneuraminic acid, 1.62 mmol/l without admixture (solid curve), with *L*-fucose, 20 mmol/l (dashed curve), and with methyl-a-*D*-mannoside, 20 mmol/l (dotted curve). Proposed procedure, for further details see methods.

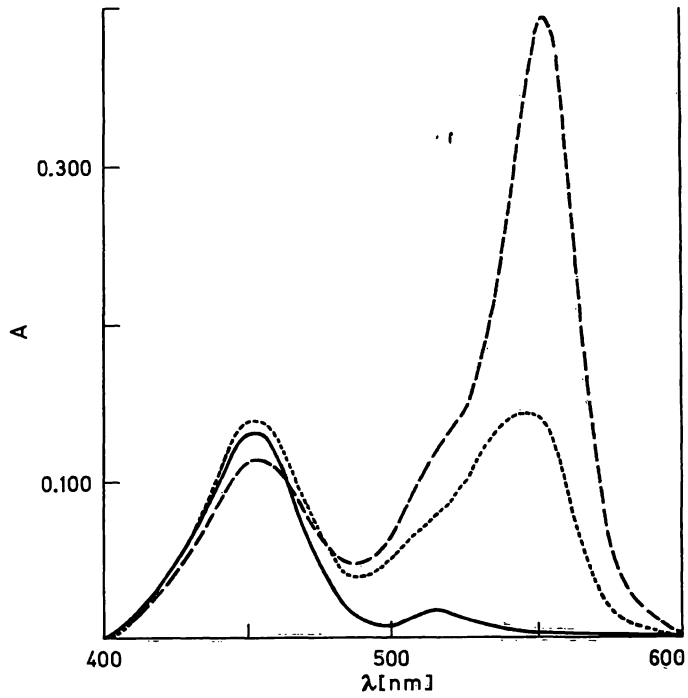

Fig. 5. Absorbance spectra of thiobarbituric acid-derived pigments from methyl-a-*D*-mannoside, 20 mmol/l (solid curve), and from cerebrospinal fluid before (dotted curve) and after hydrolysis (dashed curve) with sulphuric acid. For further details see methods.

small peak at 515 nm as depicted for methyl-α-*D*-mannoside in figure 5. Cerebrospinal fluid likewise displayed spectra with peaks at 450 nm and 550 nm, which were of almost equal intensity with native material. After hydrolysis with sulphuric acid the peak at 550 nm representing N-acetylneuraminic acid increased, while the maximum at 450 nm became lower with concomitant change of colour from orange to red (fig. 5). However, in sera, normally encountered sugar concentrations, including glucose, did not interfere with sialic acid measurement. Therefore, the chemical assay was considered a sufficiently specific and sensitive determination to be compared with recognized methods and reliable enough for the establishment of reference intervals.

In HPLC, the CV (coefficient of variation) for within-run estimates was determined to be 0.70% for the chromatographic step alone by 10 repeated injections from one hydrolysate of serum containing 2.03 mmol/l (628 mg/l) sialic acid. *Silver* et al. (22) described CVs of 2.1% (intraassay) and 5.2% (interassay) for the entire procedure. Table 4 summarizes the results of precision studies with the proposed modification and displays an equal imprecision of the chemical and the enzymatic method. These figures agreed better with data reported for the chemical microassay (3.0% intraassay, 4.3% interassay (18)) and the enzymatic test (1.0% intraassay, 1.9% interassay (19)) than with results published for *Warren*'s method (1.9% within-run, 10% day-to-day (22)).

Tab. 4. Imprecision of sialic acid assays in human sera with thiobarbituric acid and enzymatic determination (see methods).

	Thiobarbituric acid		Enzymatic test	
	mean	mean	mean	mean
Imprecision within run (n = 15)				
Sialic acid, mmol/l (mg/l)	3.25 (1010)	1.58 (490)	3.49 (1080)	1.63 (505)
CV, %	1.22	1.33	1.34	1.69
Imprecision day-to-day (n = 15)				
Sialic acid, mmol/l (mg/l)	2.57 (795)	1.28 (395)	2.67 (826)	1.25 (385)
CV, %	3.31	3.86	1.15	1.98

Intermethod comparison and reference values

Since *Silver* et al. (22) claimed higher specificity and accordingly lower values — especially at low concentrations — for the chromatographic than for the thiobarbituric acid method, we compared the results of sialic acid measurements with three methods from 72 sera. All of them showed *Gaussian* distribution (checked according to *Pearson & Hartley*) with the following ranges (mean and standard deviation): chemical assay 1.49—2.61 (2.01 ± 0.25) mmol/l, enzymatic test 1.41—2.58 (2.01 ± 0.25) mmol/l, HPLC 1.58—2.68 (2.02 ± 0.26) mmol/l. Figures 6—8 represent the close correlation of all methods. Although the regression equation of the HPLC vs. the chemical assay confirmed the aforementioned assumption of *Silver* et al. (22), higher values for the latter could be expected only for levels below 1.0 mmol/l, which never occur in serum. On the other hand, the enzymatic test throughout yielded by 3—4% lower concentrations than those of the chemical assay. This was in accordance with results of *Sugahara* et al. (19) derived from comparative determinations with the resorcinol method (28).

The same was found to be true for the enzymatic test versus HPLC, thus demonstrating the well known susceptibility of peroxidase mediated oxidative coupling reactions towards hydrogen peroxide consuming substances. The presence of 1 mmol/l (0.5 mmol/l) of some interfering compounds influenced the reaction of N-acetylneuraminic acid (3 mmol/l) as follows:

L-ascorbic acid 0.70 (0.96),
L-cysteine 0.94 (0.98),
reduced glutathione 0.80 (0.87),
2-mercaptoethanol 0.76 (0.91), and
sodium pyruvate 1.35 (1.18),

i. e. only pyruvate enhanced the amount of the red dye formed. Hence, the enzymatic measurement of sialic acid needs further evaluation.

The results of 249 determinations on sera from ostensibly healthy adults ranged from 1.53 mmol/l (472 mg/l) to 3.15 mmol/l (975 mg/l) and displayed an approximate *Gaussian* distribution in the histogram (fig. 1). According to the proposal of *Dybkaer* (29) we calculated the 0.025—0.50—0.975-reference intervals to be 1.57—1.97—2.63 mmol/l (487—610—812 mg/l) for 127 men, and to 1.69—2.08—2.64 mmol/l (524—643—817 mg/l) for 122 women. These concentrations showed no apparent dependence on age. The values for males were slightly but not significantly

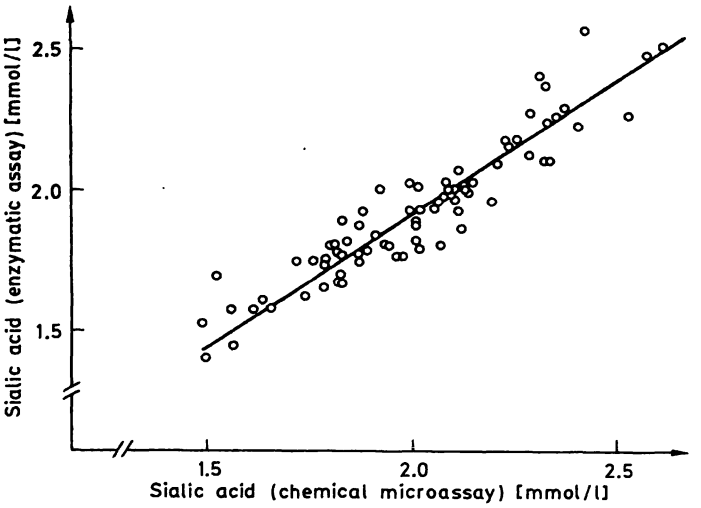

Fig. 6. Comparison of sialic acid determination by chemical microassay (abscissa) and enzymatic test (ordinate) using 78 sera: r = 0,935, y = 0.966 x — 0.010 mmol/l (standardized main component).

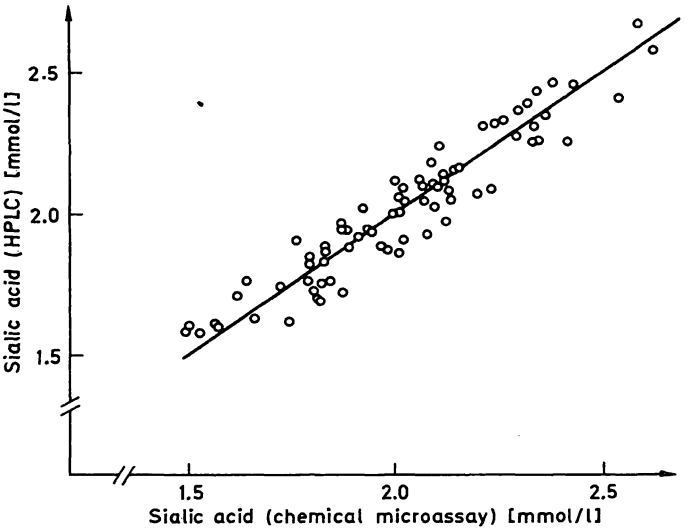

Fig. 7. Comparison of sialic acid determination by chemical microassay (abcissa) and HPLC (ordinate) using 78 sera: r = 0.949, y = 1.013 x — 0.017 mmol/l (standardized main component).

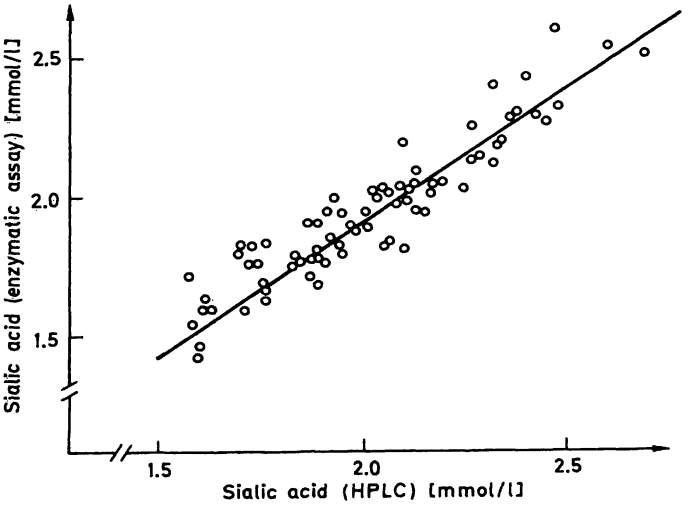

Fig. 8. Comparison of sialic acid determination by HPLC (abscissa) and enzymatic test (ordinate) using 78 sera: r = 0.931, y = 0.954 x + 0.006 mmol/l (standardized main component).

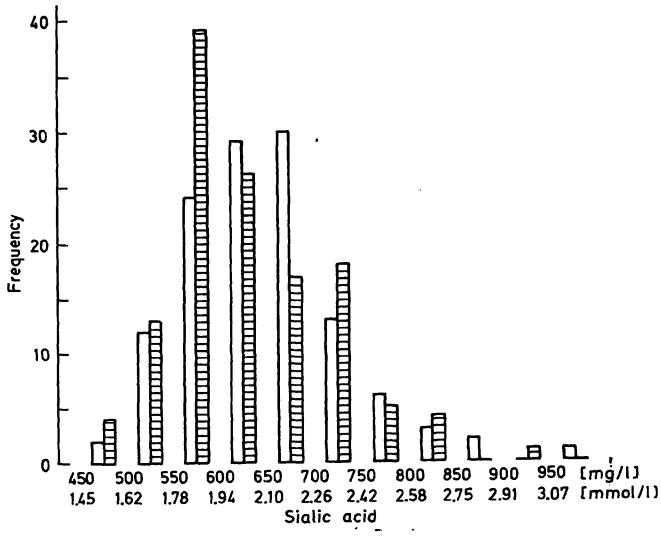

Fig. 9. Distribution of sialic acid concentrations among 249 healthy adults (127 men ▤, 122 women, □). Class interval 0.16 mmol/l (50 mg/l).

Tab. 5. Sialic acid concentrations in sera of 249 healthy adults (mean and standard deviation).

Age range, years	Concentration, mmol/l Men	n	Women	n
16–20	2.23 ± 0.34	14	2.24 ± 0.32	17
21–30	1.99 ± 0.23	37	2.11 ± 0.28	46
31–40	1.97 ± 0.22	30	2.03 ± 0.23	30
41–50	2.03 ± 0.28	29	2.01 ± 0.23	22
51–63	2.08 ± 0.29	17	2.08 ± 0.29	7
16–63	2.03 ± 0.27	127	2.09 ± 0.27	122

($p < 0.05$, z-test (32)) lower than for females (tab. 5). The data for the entire group of 249 people agreed fairly well with those given by other recent authors – apart from values reported by *Krolikowski* et al. (6) – as demonstrated in table 6.

The measurement of sialic acid in cerebrospinal fluid still remains a controversial point. The appearance of orange pigments with unhydrolysed samples has been interpreted as the reaction of free sialic acid (35), which is known to occur in meningitis by the neuraminidase activity of bacteria (36, 37). Since the origin of these chromophores (resembling the reaction of disaccharides) is not clear, we only determined the sialic acid content of cerebrospinal fluid after hydrolysis. The 2.5–50–97.5 percentiles from 43 samples were calculated to be 17.3–34.6–50.4 µmol/l (5.36–10.7–15.6 mg/l). These values coincided with the data of *Jakoby & Warren* (35), who reported 11.6 ± 5.2 mg/l as mean and standard deviation, but they surpassed those of *Saifer & Gerstenfeld* (33), who cited 5.1 ± 0.9 mg/l.

Discussion

Methods using 4-dimethylaminobenzaldehyde (20) or resorcinol (28) require the isolation of glycoproteins prior to determination. Omission of the separation step leads to erroneous results as shown above. On the other hand the thiobarbituric acid method does not require any pretreatment of the specimen. Although the oxidation of 2-oxo-3-deoxyaldonic acids and sialic acids by periodate likewise generates 2-

Tab. 6. Comparison of reference values for N-acetylneuraminic acid in serum for healthy adults (mean and standard deviation) with various methods.

Authors (Reference)	Method (Reference)	n	Reference value (mmol/l)
Krolikowski et al. (6)	Thiobarbituric acid (17)	20	0.43 ± 0.07
Böttiger & Carlson (30)	Resorcinol (28)	127	2.16 ± 0.19
Bradley et al. (7)	Diphenylamine (31)	30	2.26 ± 0.30
Macbeth & Bekesi (3)	Resorcinol (28)	41	1.88 ± 0.04
Carter & Martin (1)	Resorcinol (28)	18	2.19 ± 0.18
Saifer & Gerstenfeld (33)	Thiobarbituric acid (32)	55	2.17 ± 0.23
McNeil et al. (34)	Thiobarbituric acid (32)	25	1.71*)
Shamberger (10)	Dimethylaminobenzaldehyde (20)	134	1.74 ± 0.24
Sugahara et al. (19)	Enzymatic test (19)	24	1.94 ± 0.29
This communication	Thiobarbituric acid	249	2.06 ± 0.27

*) standard deviation not given

formylpyruvic acid as the reactive component, the preceding hydrolysis largely limits this method to sialic acids. Only the orange colour produced with native cerebrospinal fluid is evidently not derived from neuraminic acids as already noticed by *Saifer & Gerstenfeld* (33). Obviously, these pigments do not reflect the appearance of free sialic acid, but originate most probably from unhydrolysed oligosaccharides, as shown above. Periodate oxidation of disaccharides likewise generates similar chromophores with maximum absorbance at 450 nm via formation of hydroxymalonaldehyde (27). *Jakoby & Warren* (35) ascribed this colour to the reaction of deoxyribose with maximal absorbance at 532 nm, and they corrected their measurements by multiplication by a factor derived from the ratio of absorbances at 549 and 532 nm. With hydrolysed cerebrospinal fluids we always observed 549/532 ratios above 2.0 indicating that 98.5% of the absorbance at 549 nm was produced by N-acetylneuraminic acid.

There are no essential differences between the original method of *Warren* (17) and the microassays described by *Kattermann & Krieger* (18) and us. All of them uniformly require a fairly large arsenite excess over periodate for higher sensitivity of the assay as compared with *Horgan*'s (26) technique. Modifications to replace the toxic and carcinogenic arsenite by thiosulphate (25) cannot be recommended because of their high imprecision and low sensitivtiy.

The intermethod comparison reveals a close correlation, which leads to nearly identical values within the reference range, and similar precision data. Therefore, the choice of the method does not depend on analytical qualities. Although HPLC largely eliminates the impact of interfering substances, the method requires ultrafiltration and the use of an ion exclusion guard column to protect the costly analytical column. The enzymatic test is easy to perform and employs innocuous chemicals, but it is very expensive. The chemical microassay combines the advantages of simple procedure, stable reagents — only thiobarbituric acid was prepared every third day — and recycling of the extraction solvent with the disadvantage of toxic waste, which has to be preserved. Hence, we chose the chemical method to measure sialic acid for practical and economic reasons.

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